Title:

Randomized controlled study to evaluate microbial ecological effects of CPP-ACP and cranberry on dental plaque

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The results of this randomized controlled trial indicate that dentifrices containing casein phosphopeptide-amorphous calcium phosphate (CPP-ACP) and polyphenol-rich cranberry extracts were able to beneficially modulate the microbial ecology of dental plaque in a group of high caries-risk patients. This could contribute towards lowering the risk of developing new caries lesions, an important goal sought by patients, clinicians and policy makers.

ABSTRACT

Introduction: Ecological approaches to dental caries prevention play a key role in attaining long-term control over the disease and maintaining a symbiotic oral microbiome.

Objectives: This study aimed to investigate the microbial ecological effects of two interventional dentifrices: a casein phosphopeptide-amorphous calcium phosphate (CPP-ACP) dentifrice, and the same dentifrice supplemented with a polyphenol-rich cranberry extract.

Methods: The interventional toothpastes were compared with each other and with an active control fluoride dentifrice in a double-blinded randomized controlled trial. Real-time quantitative PCR (qPCR) analysis was used to determine changes in the bacterial loads of 14 key bacterial species (8 caries-associated and 6 health-associated) in the dental plaque of trial participants after they used the dentifrices for 5-6 weeks.

Results: From the baseline to the recall visit, significant differences were observed between the treatment groups in the bacterial loads of two caries-associated bacterial species *S. mutans* ($P < 0.001$) and *V. parvula* ($P < 0.001$) and three health-associated bacterial species *C. durum* ($P = 0.008$), *N. flavescens* ($P = 0.005$), and *S. sanguinis* ($P < 0.001$). Compared to the fluoride control dentifrice, the CPP-ACP dentifrice demonstrated significant differences for *S. mutans* ($P = 0.032$), *C. durum* ($P = 0.007$) and *S. sanguinis* ($P < 0.001$), while combination CPP-ACP - cranberry dentifrice showed significant differences for *S. mutans* ($P < 0.001$), *V. parvula* ($P < 0.001$), *N. flavescens* ($P = 0.003$) and *S. sanguinis* ($P < 0.001$). However, no significant differences were observed in the bacterial load comparisons between the CPP-ACP and combination dentifrices for any of the targeted bacterial species ($P > 0.05$).

Conclusions: Overall, the results indicate that dentifrices containing CPP-ACP and polyphenol-rich cranberry extracts can influence a species level shift in the ecology of the oral microbiome resulting in a microbial community less associated with dental caries.

Clinical Trial Registration: Australian New Zealand Clinical Trial Registry (ANZCTR 12618000095268).

The oral cavity harbours one of the most diverse microbiomes in the human body with over 700 different microbial species identified to date. When in equilibrium, the diversity of the endogenous oral microbial community not only prevents outgrowth of any single species or colonisation by exogenous pathogens, but also contributes to critical metabolic, physiological and immunological functions (Marsh 2018). Despite daily physical and chemical perturbations, a normal healthy microbiome exhibits remarkable long-term stability and is characterised by commensalism and mutualism with the host (Zaura et al. 2014). However, adverse local environmental conditions (e.g. frequent exposure to dietary sugars, poor oral hygiene or salivary dysfunction) can result in the breakdown of this symbiotic relationship and onset of diseases like dental caries.

Dental caries is a polymicrobial disease caused by dysbiosis in the dental plaque biofilm, with microbial community shifts driven by environmental acidification (Takahashi and Nyvad 2011). If the environmental acidic stress persists, acidogenic and aciduric bacteria will outcompete health-associated commensals and create multiple low-pH niches within the biofilm microenvironment. Health-associated commensal microorganisms can counter the acidic conditions through the production of alkaline compounds that attempt to maintain plaque pH values near neutrality (Bowen et al. 2018; Nascimento 2018). The ecological battles between the opportunistic cariogenic pathogens and the health-associated commensals will determine whether incipient lesions caused by acid-induced mineral loss from susceptible tooth surfaces will progress to cavitation or can be remineralized.

Fluoride will continue to remain the gold standard for caries prevention. However, the cariostatic effects of fluoride are predominantly due its physiochemical effects on the de-/remineralization equilibrium, suggesting the need for additional ecological measures to complement fluoride effects on the hard dental tissues. Mechanical plaque control using fluoride dentifrices alone is not likely to reverse the plaque dysbiosis responsible for the disease. Recent studies have shown that even high fluoride concentrations could not sustain antimicrobial activity against plaque biofilm metabolism (Dang et al. 2016; Souza et al. 2018). Clinical trials have further confirmed that fluoride alone was not able to effect significant changes in dental plaque microbial composition (Adams et al. 2017; Koopman et al. 2015; Reilly et al. 2016; Reilly et al. 2014). This could be one of the reasons why dental caries persists in high-risk individuals and population groups despite the widespread use of fluoride products.

A variety of antimicrobial agents have also been used in the past for caries prevention. However, the use of broad-spectrum biocides (e.g. chlorhexidine) indiscriminately eradicates even commensal bacteria that are beneficial to health. Moreover, because of their adverse side-effects, such agents may not be suitable for daily use. Once the chemotherapeutic intervention stops, susceptible tooth surfaces are often repopulated with a microbiome similar in composition to the one that was eliminated (Burne 2018). Strategies that target cariogenic virulence properties within a pathogenic biofilm, rather than broadly affecting microbial viability, would thus be preferable to conventional antimicrobials for beneficially modulating and maintaining a healthy oral microbiome.

Comprehensive caries management protocols should thus encompass agents that can influence the de-/remineralization balance as well as measures that have a moderating influence on acidogenic oral bacteria. In this regard, casein phosphopeptide-amorphous calcium phosphate (CPP-ACP) is of interest as not only can it augment the remineralizing effects of fluoride, but it can also potentially exert a beneficial effect on the microbial ecology of dental plaque (Philip and Walsh 2018). Recent *in vitro* studies have demonstrated that CPP-ACP treatment was able to effect a decrease in the abundance of caries-associated bacteria and a concomitant increase in the abundance of health-associated bacteria in multi-species biofilms grown in a cariogenic environment (Philip et al. 2019; Dashper et al. 2018). Although majority of the clinical studies on CPP-ACP have been on its remineralizing effects, several clinical studies have also reported that that regular use of CPP-ACP reduced mutans streptococci (MS) levels in saliva (Philip and Walsh 2018). This interference with cariogenic bacteria may inhibit the virulence of dental plaque and enable health-associated commensals to thrive. However, the previous studies have mostly used salivary levels of a single bacterial species (usually *Streptococcus mutans*) as the surrogate marker. Of greater relevance is the possible influence of CPP-ACP on a range of health- and disease-associated bacteria in dental plaque.

In addition, the potential exists for synergism between the milk-derived CPP-ACP and other cariogenic virulence inhibiting natural agents. Cranberry flavonoids are of particular interest as its A-linked proanthocyanidins (A-PACs) have been shown to be potent disruptors of several cariogenic virulence factors without affecting bacterial viability (Duarte et al. 2006; Gregoire et al. 2007; Koo et al. 2010, Philip et al. 2019a). The virulence attenuating effects of cranberry polyphenols have translated into reduced incidence and severity of carious lesions in an animal caries model (Koo et al. 2010). While cranberry phenols exhibit inhibitory effects against bacterial acidogenicity, aciduricity and glucan synthesis, CPP-ACP is known for its buffering and anti-adhesion effects. This suggests potential synergism if CPP-ACP and polyphenol-rich cranberry extracts are combined in a single oral care product. Hence, the objective of this study was to investigate the microbial ecological effects of a CPP-ACP dentifrice and a combination CPP-ACP – cranberry dentifrice with regards to species level changes of specific caries- and health-associated bacteria in the dental plaque of a high caries-risk group.

METHODS

Study design and Sample size

This parallel three-group double-blinded randomized controlled trial (RCT) was designed in accordance with the CONSORT guidelines. The study flow diagram is shown in Fig. 1. The trial was registered with the Australian New Zealand Clinical Trial Registry (ANZCTR 12618000095268). A clinical trial notification for using the interventional products of the study was obtained from the Australian Therapeutic Goods Administration (TGA, CT-2017-CTN-05069-1). As described previously (Lipták et al 2018), the G-POWER program tool was used to determine the sample size needed for a three-group one-way ANOVA test, assuming an alpha of 0.05, power of 80% and large effect size (f = 0.4). Based on these assumptions, the

power analysis showed that a total sample size of 66 was needed. To ensure there was enough statistical power in case some of the aforementioned assumptions were not met, and to account for attrition (from drop-outs or poor compliance), 30 subjects were recruited into each of the three trial groups.

Trial Products

Except for the active agents (CPP-ACP and cranberry extract) in the interventional toothpastes, all the trial toothpastes had same ingredients and a standard 1100 ppm fluoride concentration. The interventional toothpastes were compared with each other and with an active control fluoride dentifrice. Details of the three treatment groups are as follows:

- CPP-ACP group: dentifrice containing 10% (w/v) CPP-ACP (MI Paste® ONE, GC Corporation, Tokyo, Japan).
- All-in-One group: 0.25% (w/w) cranberry extract (Diana Food, Champlain, QC, Canada) was incorporated into the CPP-ACP dentifrice. The polyphenol-rich cranberry extract was a highly purified, organic, sugar-free natural extract that contained more than 80% A-PACs.
- Active control group: standard fluoride dentifrice (GC Corporation), which had the same vehicle and flavour as the interventional dentifrices.

Blinding and Randomization

The trial dentifrices were coded and had the same external packaging, with their content not revealed to the participants or the enrolling investigator. The dentifrice product codes were known only to the chief supervisor of the study (L.J.W) who was not involved in participant enrolment, product allocation or data analysis. The product codes were broken only after the final analysis of the study results. The randomization allocation list was made by a single investigator (N.P) in SPSS v 24 (IBM, New York, NY, USA) using the random number generation function that equally distributed a third of the 90 trial subjects to each of the three coded trial groups. The same investigator enrolled participants and allocated the trial interventions to the study participants, without being aware of which dentifrice the individual trial participants had received.

Participants and Eligibility Criteria

The study protocol was approved by the institutional human research ethics committee (HREC/18/QPCH/7). The participants were child/teenage patients recruited from the orthodontic clinics of the University of Queensland School of Dentistry from July 2018 to October 2018.

The inclusion criteria for study participants were: (i) minimum of 10 years of age with at least 4 fully erupted permanent maxillary teeth; (ii) undergoing fixed orthodontic treatment in both arches with treatment having been underway for at least 1 month; (iii) not currently using antibiotics/antimicrobial mouth rinses; and (iv) available to attend a recall appointment in 5-6 weeks. The exclusion criteria were: (i) any medical condition or disability preventing self-tooth brushing; (ii) allergy to milk casein proteins or

benzoate preservatives present in the CPP-ACP toothpaste; (iii) unwillingness to use a fluoridated toothpaste; and (iv) untreated periodontal disease or clinical evidence of active caries. All the study participants were given standardized oral hygiene instructions and encouraged to use the allocated toothpaste for routine twice-daily tooth brushing throughout the trial period. Participant compliance with using the dentifrice was assessed based on the weight of the toothpaste tube at the end of the trial.

Dental Plaque Sample Collection

Dental plaque samples were collected from the study subjects at two time points - at a baseline visit just before the participants started using the trial toothpastes, and then at a recall visit scheduled after 5-6 weeks of using the allocated toothpastes. The plaque samples were collected by using a sterile microbrush to swab the labial and gingival third of the upper and lower anterior teeth. Sample were collected without removing any orthodontic modules, chain or ligature-ties. The microbrush tips were placed in 0.01% thiomersal (Sigma-Aldrich, St. Louis, MO, USA) solution and stored at -80°C until microbial analysis.

Microbial Analysis

The bacterial load of 14 selected bacterial species was determined using real-time quantitative PCR (qPCR) analysis. The bacterial panel included 8 caries-associated bacterial species (*Actinomyces gerensceriae*, *Lactobacillus gasseri*, *Streptococcus mutans*, *Streptococcus sobrinus*, *Streptococcus parasanguinis*, *Scardovia wiggsiae*, *Veillonella parvula* and *Provotella denticola*), and 6 health-associated commensal bacterial species (*Streptococcus sanguinis*, *Streptococcus mitis*/*oralis*, *Steptococcus salivarius*/*thermophilus*, *Corynebacterium durum*, *Rothia aeria*/*dentocariosa* and *Neisseria flavescens*)

DNA extraction

DNA was extracted from the plaque samples using the MO BIO Power Soil DNA Isolation Kit (MO BIO Laboratories, Carlsbad, CA, USA) according to manufacturer's instructions. RNA was removed using RNAse A (Thermo Fischer Scientific, Scoresby, Australia) and the amount of isolated DNA quantified spectrophotometrically.

16S rRNA sequencing

The bacterial load of the 14 selected bacterial species was determined using a custom-made qPCR array (16 x 24 format; Qiagen, Hilden, Germany). The assay used the 16S rRNA gene of the relevant bacterium, with the proprietary probes designed using the GreenGene version 13.8 database (DeSantis et al. 2006) for 16S sequences. Each DNA sample was mixed with a proprietary master mix and robotically dispensed into a 384-well plate (10µL, 7 ng DNA/well) containing freeze-dried primers and fluorogenic probes for each of the bacterial 16S rRNA genes tested. Arrays also contained a positive PCR control to test for inhibitors in the sample, and a non-template control to account for assay background. Reactions were performed with the 384-well plate QuantStudio™ 6 Flex Real-Time PCR sequence detection system (Thermo

Fisher Scientific) with the following cycling conditions: enzymatic activation at 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec, and 60°C for 2 min. Data was analysed using the sequence detection system software (QuantStudio v1.3).

Primary outcome

The study evaluated the fold changes effected by the trial toothpastes in the bacterial load of the 14 key bacterial species in the dental plaque of trial participants from the baseline to the recall visit.

Statistical analysis

The fold change of each bacterial amplicon was calculated using the comparative cycle threshold method (ΔΔCt). Briefly, for each sample, the Ct value of each bacterial target was normalized to the mean Ct value of all bacterial species ($\Delta Ct^{\text{bacteria species}} = Ct^{\text{bacteria species}} - Ct^{\text{mean of all bacterial species}}$). The trial group ΔΔCt for each bacterial species was then calculated as follows: $\Delta\Delta Ct^{\text{trial group}} = \Delta Ct^{\text{bacterial species (recall visit)}} - \Delta Ct^{\text{bacterial species (baseline visit)}}$. Fold increase, or decrease, in bacterial abundance from the baseline to recall visit for each trial group was calculated based on the formula: $2^{-\Delta\Delta Ct}$. For each bacterial species, one-way ANOVA was used to test for differences among the three trial groups using the ΔΔCt values (Yuan et al. 2006). To adjust for the multiple bacterial comparisons, *P*-values were considered significant only if they were less than the Simes critical *P*-value (Simes 1986). For bacterial species that showed significant ANOVA results, paired t-tests were performed and the Bonferroni correction used to assess for any significant differences between the treatment group pairs.

RESULTS

At the baseline visit, there was no significant differences in the prevalence of any of the bacterial species across the treatment groups ($P > 0.05$). Except for 4 bacterial species (*L. gasseri*, *S. sobrinus*, *S. parasanguinis* and *S. wiggsiae*), all the other 10 bacterial species were highly prevalent in all groups at the baseline visit (Appendix Table 1). Age and gender distribution were also comparable among the three trial groups (Appendix Table 2).

The relative abundance of cariogenic and health-associated bacterial species at the baseline and recall visit, and the subsequent fold change in bacterial loads over time, are presented in the Appendix Tables 3 and 4 respectively. From the baseline to the recall visit, the treatment groups differed significantly in the mean changes in bacterial loads of two caries-associated species *V. parvula* ($P < 0.001$) and *S. mutans* ($P < 0.001$) (Table 1, Fig. 2A) and three health-associated bacterial species *C. durum* ($P = 0.008$), *N. flavescens* ($P = 0.005$), and *S. sanguinis* ($P < 0.001$) (Table 1, Fig. 2B). Post-hoc analysis revealed that the CPP-ACP group differed significantly from the control group with respect to mean changes in loads of *S. mutans* ($P = 0.032$), *C. durum* ($P = 0.007$) and *S. sanguinis* ($P < 0.001$), while the All-in-One group differed from the control group with respect to mean changes in loads of *S. mutans* ($P < 0.001$), *V. parvula* ($P < 0.001$), *N. flavescens* ($P = 0.003$) and *S. sanguinis* ($P < 0.001$) (Table 2). Bacterial loads of *V. parvula* and *S. mutans* decreased over time,

both in the CPP-ACP group (mean fold decreases of 0.31 and 0.52 respectively) and in the All-in-One group (mean fold decreases of 0.11 and 0.23 respectively), and increased over time in the control group (mean fold increases of 1.10 and 2.51 respectively) (Table 1, Fig. 2A). Conversely, bacterial loads of *N. flavescens* and *S. sanguinis* increased over time in the CPP-ACP group (mean fold increases of 2.05 and 2.92 respectively) and in the All-in-One group (mean fold increases of 12.27 and 3.44 respectively), and decreased over time in the control group (mean fold decreases of 0.64 and 0.46 respectively). For *C. durum,* there were fold increases of 14.45, 6.11, and 1.92 in the CPP-ACP, All-in-One, and control groups respectively (Table 1, Fig. 2B).

Compared to the CPP-ACP group, the All-in-One group generally showed greater mean fold decreases over time in the loads of the caries-associated bacteria, and higher mean fold increases over time in the loads of the health-associated bacteria (except for *C. durum*). However, these differences were not statistically significant ($P > 0.05$) (Table 2). Mean changes in the loads of the other 9 bacterial species assessed did not differ between the treatment groups ($P > 0.05$) (Table 1).

DISCUSSION

This clinical trial provides evidence that a CPP-ACP dentifrice can beneficially influence dental plaque microbial composition in a high caries-risk group. Specifically, we demonstrate species level changes in dental plaque after using dentifrices containing CPP-ACP with regards to two caries-associated bacterial species and three health-associated bacterial species. The addition of a highly purified polyphenol-rich cranberry extract to the CPP-ACP dentifrice gave a small enhancement to the microbial ecological effects of CPP-ACP, with the CPP-ACP – cranberry dentifrice significantly reducing *V. parvula* bacterial loads and significantly increasing *N. flavescens* bacterial loads compared to the fluoride control, effects that were not seen with the dentifrice containing CPP-ACP alone. This observation, combined with the fact the CPP-ACP – cranberry dentifrice effected a greater fold decrease in *S. mutans* levels and a higher fold increase in *S. sanguinis* levels than the CPP-ACP dentifrice, suggests the possibility that greater beneficial effects from the combination dentifrice could be seen if it was used for longer period, or a higher concentration of cranberry extract was used.

The proposed mechanisms for the microbial ecological effects of CPP-ACP observed in this study could be related to its buffering and anti-adhesion effects. Various clinical studies have reported that regular use of CPP-ACP topical crèmes had pronounced buffering influences on salivary and plaque pH (Philip and Walsh 2018). The buffering effects of CPP-ACP are due to its ability to act as a reservoir of peptides and phosphate ions. The catabolism of peptides by plaque peptidases would buffer against a pH fall through the production of ammonia (Reynolds and Riley 1989). CPP phosphoseryl residues that are more resistant to hydrolysis can readily accept protons and neutralize plaque acids (Reynolds 1987). Furthermore, the inorganic phosphate (PO_4^{3-}) and organic phosphate ($-O-PO_3^{2-}$) ions in the CPP-ACP nanocomplex also contribute to elevating salivary/plaque pH (Dashper et al. 2018). The buffering actions of CPP-ACP can result in a homeostatic oral environment that favours the growth of health-associated commensals. Besides its buffering effects, CPP-ACP is also known to inhibit bacterial co-aggregation and accumulation in dental

plaque by binding to bacterial surfaces (with a strong affinity for *S. mutans*) and pellicle macromolecules (Reynolds et al. 2003; Rose 2000). In the present study, the CPP-ACP dentifrice effected a decrease in the bacterial load of *S. mutans* and an increase in bacterial loads of *C. durum* and *S. sanguinis.* These results can possibly be attributed to the anti-adhesion and buffering influences of CPP-ACP on the plaque biofilm.

Polyphenol-rich cranberry extracts and purified cranberry A-PACs have demonstrated potent inhibitory effects against *S. mutans* glycolytic, F_1F_0-ATPase and glucosyltransferase (Gtf) enzymes, while also disrupting the structural architecture of *in vitro* biofilms (Duarte et al. 2006; Gregoire et al. 2007, Koo et al. 2010, Philip et al. 2019a; 2019b). The ability of cranberry polyphenols to influence key cariogenic virulence properties without affecting microbial viability make them potentially ideal agents to lower cariogenic virulence and improve the microbial ecological balance of dental plaque. Considering that the combination dentifrice showed the highest fold decreases in the cariogenic *S. mutans* and *V. parvula* bacterial species, suggests that the virulence-inhibitory effects of the polyphenol-rich cranberry extract extended to a clinical environment too.

The two bacterial species (*S. mutans* and *V. parvula*) that showed significant decreases in their bacterial loads have both been strongly associated with dental caries. *S. mutans*, even when present in low numbers, is still considered a keystone pathogen in the disease process as it is largely responsible for the initial assembly of the cariogenic glucan-rich biofilm matrix (Bowen et al. 2018). Other acidogenic and aciduric bacteria lack the specific Gtfs to synthesize insoluble glucans that are considered the foundational building blocks of cariogenic biofilms (Hajishengallis et al. 2017). The *S. mutans*-derived Gtf exoenzymes orchestrate the virulent EPS glucan-rich matrix scaffold, paving the way for other resident aciduric bacteria (e.g. lactobacilli, bifidobacteria, *Scardovia* spp. etc.) to dominate the microbiome as the plaque biofilm matures (Bowen et al. 2018; Burne 2018). *Veillonella*, an obligate anaerobic Gram-negative bacterium, is considered a bridge organism in the biofilm, and is commonly isolated from initial lesions (Aas et al. 2005; Gross et al. 2012). It was earlier perceived that presence of this bacterium in plaque may be beneficial as it neutralizes acidic pH by utilizing lactic acid as its nutritional source of energy. However, evidence from recent microbiome studies confirm that *Veillonella* is strongly associated with caries lesions (Jiang et al. 2016; Richards et al. 2017; Tanner et al. 2016; Zhou et al. 2016). *Veillonella* was seen to promote *S. mutans* growth even in the presence of antagonistic *Streptococcus gordonii*, while the acetic acid produced from *Veillonella* lactate catabolism can also demineralize enamel (Bowen et al. 2018). Metatranscriptomic studies reveal that *Veillonella* is positively stimulated by low-pH conditions and exhibits intracellular pH control mechanisms (Do et al. 2015; Edlund et al. 2015). Furthermore, even aciduric organisms show reduced growth when pH drops below 4.5, and *Veillonella* can help the acidogenic microbial community by maintaining a relatively more neutral pH (Tanner et al. 2016).

In the present study, three health-associated bacterial species (*S. sanguinis, C. durum* and *N. flavescens*) showed significant increases in their bacterial loads from the baseline to the recall visit. These commensals have various strategies to foster an oral biofilm environment that discourages the emergence

and dominance of opportunistic cariogenic pathogens. *S. sanguinis* belongs to a group of oral commensals known to possess the arginine deaminase system (ADS) that can neutralize acids produced from carbohydrate metabolism by generating ammonia from prebiotic dietary and salivary substrates (Nascimento 2018). The ammonia produced not only alkalizes the bacterial cytoplasm but also gets released outside the cell promoting an overall elevation in plaque pH. This creates an environment that supports the growth of other acid-sensitive commensals. Moreover, endogenous ammonia production promotes stability of health-associated biofilms by affording bioenergetic advantages to the commensal bacteria (Burne 2018). The bacterial species *C. durum* has an established nitrate (NO_3^-) reductase system which rapidly reduces salivary NO_3^- to nitrite (NO_2^-) as part of its respiration. In a low pH environment, NO_2^- dissociates to form a range of nitrogen oxides, most notably nitric oxide (NO). The NO_3^--NO_2^--NO reduction pathway can limit the growth of cariogenic bacteria because of the antimicrobial effects of NO (Doel et al. 2004). *Neisseria* is a catalase-positive aerobic species that has been identified as part of the healthy "core microbiome" of the human oral cavity (Zaura et al. 2009). *Neisseria flavescens* is known to be asaccharolytic and has been associated with caries-free status in next-generation sequencing studies of saliva and plaque samples (Gross et al. 2012; Richards et al. 2017)

Prudency is required when interpreting the results of this study. Further studies are required to know whether the beneficial plaque microbial changes shown here can translate into lowering the caries increment in a high-risk population group, which should be the only criteria to judge the efficacy of any anti-caries agent. While the microarray and taxon-specific qPCR techniques employed in this study allowed quantification of multiple high-interest bacterial species from dental plaque, it does not make an evaluation of the numerous other microorganisms present in dental plaque. Moreover, the taxonomic composition of plaque samples alone does not give complete information about the functional output of plaque microbial communities or their role in health and disease. Future research using metagenomic and metatranscriptomic analysis tools can provide genetic information of the whole microbial community and provide a better understanding of the metabolic output and functional profile of oral microbial communities.

There is now evidence that routine provision of interventions that deliver small but relevant benefits can support the maintenance of a symbiotic oral microbiome (Marsh 2018). A recent RCT demonstrated that that a toothpaste containing enzymes and proteins promoted an overall community shift by increasing the relative abundance of bacteria associated with gingival health and decreasing those associated with periodontal disease (Adams et al. 2017). The rationale for applying similar ecological approaches to dental caries prevention is apparent, and various strategies are currently under investigation (Philip et al. 2018a). This study suggests the possibility of using milk-derived CPP-ACP and polyphenol-rich cranberry extracts in daily use oral care products to ecologically modulate plaque microbial communities.

Overall, the results of this clinical study indicate CPP-ACP delivered in a dentifrice exerts useful microbial ecological effects, and this is enhanced somewhat by including a polyphenol-rich cranberry extract as well. In the past two decades, CPP-ACP topical crèmes and chewing gums have been in use globally for its

remineralizing actions. The present study shows that twice-daily brushing with a CPP-ACP dentifrice can provide an additional ecological benefit that could potentially translate into lowering caries-risk. Further studies will be needed to resolve whether polyphenol-rich cranberry extracts or other additives can enhance the ecological effects of CPP-ACP. Finally, it should be emphasized that microbial ecology-based therapeutics are only one component within a holistic caries preventive strategy that should primarily aim to reverse the environmental stresses responsible for the dental plaque dysbiosis.

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Figure Legends

Fig. 1 Study flow diagram

Fig. 2 Mean fold change of A) caries-associated and B) health-associated bacterial species from the baseline to recall visit. Ratio below 1 indicates lower relative abundance; a ratio above 1 indicates higher relative abundance. * indicates statistically significant results. *P*-values in brackets are from the one-way ANOVA tests

Table Legends

Table 1 Comparison of treatment groups with regard to mean fold changes ($2^{-\Delta\Delta Ct}$) in bacterial loads of *caries-associated* and *health-associated* bacterial species from the baseline to recall visit

Table 2 Post-hoc pairwise comparisons between treatment groups